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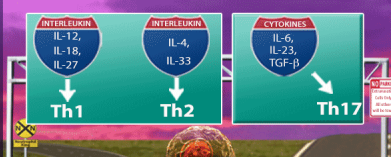
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## **Differential Effects of CpG DNA on IFN- $\beta$ Induction and STAT1 Activation in Murine Macrophages versus Dendritic Cells: Alternatively Activated STAT1 Negatively Regulates TLR Signaling in Macrophages**

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# Differential Effects of CpG DNA on IFN- $\beta$ Induction and STAT1 Activation in Murine Macrophages versus Dendritic Cells: Alternatively Activated STAT1 Negatively Regulates TLR Signaling in Macrophages<sup>1</sup>

Kate Schroder,\* Martina Spille,<sup>†</sup> Andreas Pilz,<sup>‡</sup> Jane Lattin,\* Konrad A. Bode,<sup>†</sup> Katharine M. Irvine,\* Allan D. Burrows,\* Timothy Ravasi,<sup>§</sup> Heike Weighardt,<sup>¶</sup> Katryn J. Stacey,\*<sup>||</sup> Thomas Decker,<sup>‡</sup> David A. Hume,\* Alexander H. Dalpke,<sup>†</sup> and Matthew J. Sweet<sup>2\*||</sup>

Classical STAT1 activation in response to TLR agonists occurs by phosphorylation of the Y701 and S727 residues through autocrine type I IFN signaling and p38 MAPK signaling, respectively. In this study, we report that the TLR9 agonist CpG DNA induced *Ifn- $\beta$*  mRNA, as well as downstream type I IFN-dependent genes, in a MyD88-dependent manner in mouse myeloid dendritic cells. This pathway was required for maximal TNF and IL-6 secretion, as well as expression of cell surface costimulatory molecules. By contrast, neither A- nor B-type CpG-containing oligonucleotides induced *Ifn- $\beta$*  in mouse bone marrow-derived macrophages (BMM) and a CpG-B oligonucleotide did not induce *Ifn- $\beta$*  in the macrophage-like cell line, J774. In BMM, STAT1 was alternatively activated (phosphorylated on S727, but not Y701), and was retained in the cytoplasm in response to CpG DNA. CpG DNA responses were altered in BMM from STAT1<sup>S727A</sup> mice; *Il-12p40* and *Cox-2* mRNAs were more highly induced, whereas *Tlr4* and *Tlr9* mRNAs were more repressed. The data suggest a novel inhibitory function for cytoplasmic STAT1 in response to TLR agonists that activate p38 MAPK but do not elicit type I IFN production. Indeed, the TLR7 agonist, R837, failed to induce *Ifn- $\beta$*  mRNA and consequently triggered STAT1 phosphorylation on S727, but not Y701, in human monocyte-derived macrophages. The differential activation of *Ifn- $\beta$*  and STAT1 by CpG DNA in mouse macrophages vs dendritic cells provides a likely mechanism for their divergent roles in priming the adaptive immune response. *The Journal of Immunology*, 2007, 179: 3495–3503.

Activation of innate immune cells such as macrophages and dendritic cells (DC)<sup>3</sup> commonly involves concerted signaling by cytokines such as IFNs, and microbial products such as LPS and CpG DNA (1). Many cytokines act

through members of the hemopoietin receptor family, and their actions are coupled to the JAK-STAT signaling cascade. Microbial products act through the TLR family, which are coupled via adaptor proteins such as MyD88 to downstream effectors such as NF- $\kappa$ B. IFNs prime for maximal activation in response to the TLR agonists LPS and CpG DNA, and the IFN-responsive STAT family member STAT1 can integrate the two pathways; IFNs and LPS both induce STAT1 phosphorylation and STAT1-dependent gene expression (1, 2).

TLRs differ in the use of specific adaptors and consequent induction of target genes. The response to LPS requires TLR4 (3), which signals through both MyD88-independent (mediated by the TIR domain-containing adaptor proteins 1 and 2 and MyD88-dependent pathways (reviewed in Ref. 4). By contrast, CpG DNA-dependent signaling via TLR9 absolutely requires MyD88 (5). Consequently, LPS, but not CpG DNA, induces a set of MyD88-independent genes in macrophages (6, 7). One of the MyD88-independent genes induced by LPS in macrophages encodes the type I IFN, IFN- $\beta$  (8, 9). LPS-induced IFN- $\beta$  acts in an autocrine fashion to activate tyrosine phosphorylation of STAT1, which in turn induces a set of target genes, including inducible NO synthetase in macrophages (9, 10).

TLR signaling also modifies STAT1 more directly through phosphorylation at S727 (11, 12), which occurs via a p38 MAPK-dependent pathway (13–15). The contributions of S727 phosphorylation to the actions of STAT1 were analyzed using STAT1<sup>S727A</sup> mutant mice, in which an alanine is substituted for the serine at residue 727 of STAT1 (12). These mice were resistant to LPS

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; BMM, bone marrow-derived macrophage; HMDM, human monocyte-derived macrophage; IP-10, IFN- $\gamma$ -inducible protein-10; IRF, IFN regulatory factor; LTA, lipoteichoic acid; pDC, plasmacytoid DC; qPCR, quantitative PCR; WT, wild type.

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shock, and macrophages from these mice were less sensitive to the combined effects of IFN- $\gamma$  plus LPS in vitro (12). Phosphorylation of STAT1 on Y701 is required for homo- and heterodimerization and for nuclear translocation and transcriptional activation. Nevertheless, STAT1 phosphorylated at S727, but not Y701, can still regulate gene expression (16–20). STAT1 serine phosphorylation was required, whereas tyrosine phosphorylation and homodimerization were dispensable, for maximal constitutive expression of caspase-2 and -3 in fibroblasts (16). Furthermore, Y701 mutants of STAT1 still regulated the constitutive expression of several genes, including *CycA*, *Hsp70*, and *Lmp2*, in fibroblasts (17).

Early studies on CpG DNA signaling in macrophages implied that this stimulus did not elicit type I IFN production (21, 22), consistent with the inability of CpG DNA to activate MyD88-independent signaling pathways (5). However, recent reports demonstrated that CpG DNA triggered IFN- $\beta$  production in myeloid DC in a MyD88-dependent fashion via IFN regulatory factor (IRF)1 (23, 24). In addition, in plasmacytoid DC (pDC), endosomal retention of CpG DNA enabled MyD88-dependent activation of the IRF family member, IRF7, leading to type I IFN production (25, 26). CpG-containing oligonucleotides that effectively induce type I IFN in pDC are termed A type and have a palindromic central CpG motif, which can form a double-stranded region, and strings of G residues at both the 3' and 5' ends. A-type and conventional single-stranded B-type CpG-containing oligonucleotides traffic differentially in pDC; A-type oligonucleotides are retained in early endosome-like structures and initiate signaling leading to type I IFN production, whereas B-type oligonucleotides traffic to a late endosome/lysosome compartment and more strongly induce inflammatory cytokines, such as TNF (26). Differences between macrophages and DC in their response to MyD88-dependent signals could be important in constraining pathological consequences of macrophage activation (e.g., septic shock), while permitting activation of acquired immunity.

In this study, we confirm that macrophages differ from DC in their response to MyD88-dependent signaling by CpG DNA. The MyD88-dependent production of IFN- $\beta$  was essential for CpG DNA-mediated DC maturation, and provides an important insight into the powerful adjuvant activity of CpG DNA. Conversely, the failure of macrophages to respond to CpG DNA with inducible IFN- $\beta$  expression reveals a novel cytoplasmic function for STAT1, which limits the production of a subset of inflammatory mediators. We propose that the divergent responses of macrophages and DC to CpG DNA could derive from differential expression of members of the IRF family.

## Materials and Methods

### General reagents

Recombinant mouse IFN- $\gamma$  (R&D Systems) and IFN- $\beta$  (PBL) were used at a final concentration of 500 pg/ml and 100 U/ml, respectively. LPS (from *Salmonella minnesota*; Sigma-Aldrich) was used at a final concentration of 10 ng/ml, unless otherwise stated. Lipoteichoic acid (LTA) from *Staphylococcus aureus* was a gift from S. Morath (Konstanz, Germany); PAM<sub>3</sub>CSK<sub>4</sub> was obtained from EMC Microcollections. R837 (imi-quimod), a specific TLR7 agonist (27, 28), was purchased from InvivoGen. Human rCSF-1 (a gift from Chiron) was used at a final concentration of  $1 \times 10^4$  U/ml (100 ng/ml). Unless otherwise stated, the well-characterized B-type phosphorothioate-modified oligonucleotide 1668S (5'-tccatgagcttcctgatgct-3') (29) was used in all experiments at a final concentration of 0.1  $\mu$ M. The GC inversion of this oligonucleotide, 1668S-GC, 5'-tccatgagcttcctgatgct-3', was used to control for CpG DNA-independent effects on cells (29). In experiments in which A-type oligos were examined, the D19 oligonucleotide (5'-ggTGCATCGATGCAgggggG-3') (26) and the 1585 oligonucleotide (5'-ggGGTCAACGTTGAgggggG-3') (30) were used. Upper and lower case letters indicate bases with phosphodiester- and phosphorothioate-modified linkages, respectively. All oligonucleotides were synthesized by Geneworks. Abs used in Western blotting were purchased

from Cell Signaling Technology (anti-STAT1, anti-phospho-Y701-STAT1, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK-1/2, anti-phospho-ERK-1/2, anti-CSF-1R, goat anti-rabbit IgG), Calbiochem (anti-phospho-S727-STAT1), or Santa Cruz Biotechnology (anti-PU.1). All primary Abs were used at 1:1000, except anti-PU.1, which was used at 1:5000, whereas the secondary Ab was used at 1:3000.

### Preparation of macrophages and DC

Bone marrow-derived macrophages (BMM) were obtained by ex vivo differentiation from mouse bone marrow progenitors in the presence of rCSF-1 over 7 days, as described previously (31, 32). C57BL/6 male mice (6–8 wk old) kept under specific pathogen-free conditions were used in BMM generation. For experiments with wild-type (WT) vs STAT1<sup>S727A</sup> mice, bone marrow progenitors were pooled from equal numbers of age-matched male and female mice in each experiment. J774 macrophages (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 5% FCS and antibiotics (100 IU/ml penicillin G and 100 IU/ml streptomycin sulfate). GM-CSF-derived (CD11c<sup>+</sup>, B220<sup>−</sup>) bone marrow-derived DC (BMDC) were prepared, as described previously (33), from C57BL/6 female 6- to 8-wk-old mice. Where the effect of IFNAR1 or MyD88 genotype was examined, SV129 IFNAR1<sup>+/+</sup>, SV129 IFNAR1<sup>−/−</sup>, BALB/c MyD88<sup>+/+</sup>, or BALB/c MyD88<sup>−/−</sup> mice were used as a source of DC and BMM. All experiments with mouse primary cells were conducted in accordance with local animal ethics guidelines.

Human macrophages were derived from monocytes isolated from blood donations obtained from the Australian Red Cross Blood Bank. PBMC were separated from buffy coat by Ficoll-Paque (Amersham) density centrifugation. CD14<sup>+</sup> monocytes were isolated from the PBMC by positive selection for CD14 using MACS magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions. Monocytes were seeded on tissue culture plastic at  $15 \times 10^6$  per plate in IMDM (Invitrogen Life Technologies) with 10% FCS, CSF-1, and antibiotics, as above, and allowed to differentiate into macrophages for 6 days. Macrophages were supplemented with CSF-1-replete medium on day 5, and both the adherent and nonadherent cells were reseeded on day 6 and harvested for analysis on day 7.

### Determination of mRNA expression by quantitative PCR (qPCR)

Cellular RNA was extracted using RNeasy kits (Qiagen) or HighPure RNA kits (Roche). Contaminating genomic DNA was removed using DNA-Free (Ambion), and cDNA was synthesized using Superscript III (Invitrogen Life Technologies) or reverse transcriptase (MBI Fermentas) and oligo(dT) primer. Negative control reactions were performed as above, with the omission of the enzyme. cDNA was quantitated using the Platinum SYBR Green quantitative PCR system (Invitrogen Life Technologies) or TaqMan reagents (AbGene) and fluorogenic probes (FAM/TAMRA; Eurogentec). Negative control reactions were cycled alongside test samples to ensure the absence of contaminating genomic DNA. Data were analyzed using ABI Prism software. Expression was determined relative to the abundance of housekeeping genes (SYBR Green, hypoxanthine guanine phosphoribosyl-transferase *Hprt*; TaqMan,  $\beta$ -actin). Transcript abundance (*Gene/Hprt* or *Gene/ $\beta$ -actin*) and SD were determined, as recommended by Applied Biosystems. Where inducible expression in cells of different genotypes was compared, expression was determined as a fold induction relative to basal expression within the genotype. Profiles were quantitated in at least three separate experiments using completely independent preparations of cells and RNA extracts, unless otherwise stated. Real-time PCR primer pairs were designed and tested as described previously (34). Primer sequences are available on request.

### ELISA

The levels of secreted cytokines (IFN- $\gamma$ -inducible protein-10 (IP-10), TNF, and IL-6) in culture supernatants were determined using ELISA kits (OptEia; BD Biosciences).

### Flow cytometry

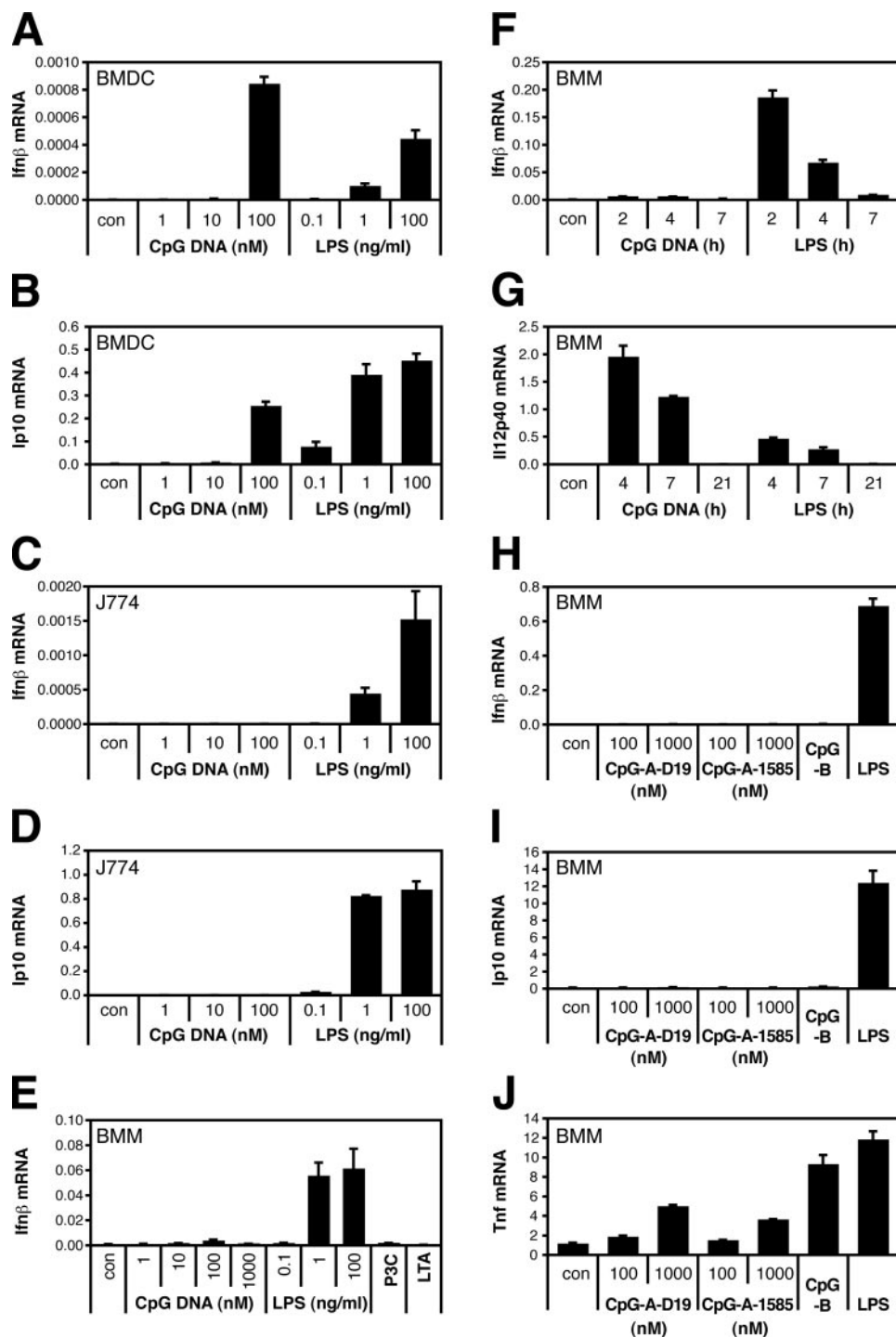
Cell surface proteins were stained using anti-CD80 and anti-CD86 Abs (BD Biosciences) and analyzed by flow cytometry using a FACS Canto (BD Biosciences).

### Immunoblot analysis

Western analysis of phospho-specific protein forms included phosphatase inhibitors (1 mM sodium vanadate, 1 mM sodium pyrophosphate, 1 mM sodium molybdate, 10 mM sodium fluoride) in all steps of protein extract



**FIGURE 1.** CpG DNA induces *Ifn- $\beta$*  and IFN- $\beta$ -dependent gene expression in BMDC, but not macrophages. BMDC (A and B), J774 cells (C and D), and BMM (E) were stimulated with varying doses of CpG DNA or LPS for 3 h. Experiments with BMM also included stimulation with 5  $\mu$ g/ml PAM<sub>3</sub>CSK<sub>4</sub> (P3C) or 10  $\mu$ g/ml LTA. Total cellular RNA was prepared, and the mRNA expression of *Ifn- $\beta$*  (A, C, and E) and *Ip-10* (B and D), relative to  $\beta$ -actin (A–E) was determined by qPCR. Data points represent the average of duplicates + SD. Profiles are representative of two independent experiments. F and G, BMM were stimulated with CpG DNA (0.1  $\mu$ M) or LPS (10 ng/ml) over a time course (F and G). Total cellular RNA was prepared, and mRNA expression of *Ifn- $\beta$*  (F) and *Il-12p40* (G) relative to *Hprt* was determined by qPCR. H–J, BMM were stimulated with 0.1  $\mu$ M or 1  $\mu$ M A-type CpG DNA (D19, 1585), B-type CpG DNA (1668S, 0.1  $\mu$ M), or LPS (10 ng/ml) for 2 h. Total cellular RNA was prepared, and mRNA expression of *Ifn- $\beta$*  (H), *Ip-10* (I), and *Tnf* (J) relative to *Hprt* was determined by qPCR. Data points represent the average of triplicates + SD. Profiles are representative of at least three independent experiments.



preparation and immunodetection. The fractionation of nuclear and extranuclear proteins was performed at 4°C, and all solutions for extract preparation were prechilled and included 5  $\mu$ M PMSF and 1 $\times$  protease inhibitors (Roche). Cells were harvested and washed twice with PBS. The cell pellet was resuspended in 225  $\mu$ l of plasma membrane lysis buffer (10 mM HEPES (pH 7.4), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.1% Nonidet P-40) per  $10 \times 10^6$  cells, and incubated for 5 min. Nuclei were separated from the solubilized extranuclear extract by centrifugation, and the nuclei were washed three times with plasma membrane lysis buffer. Nuclei were resuspended in 75  $\mu$ l of nuclei lysis buffer (20 mM HEPES (pH 7.8), 420 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1.5 mM MgCl<sub>2</sub>) per  $10 \times 10^6$  cells and incubated for 10 min. The nuclear extract was clarified by centrifugation. Total protein in whole cell, nuclear, and extranuclear extracts was quantitated using the BCA Protein Assay Kit (Pierce) to normalize total protein loading. Whole cell extracts were prepared by boiling lysis

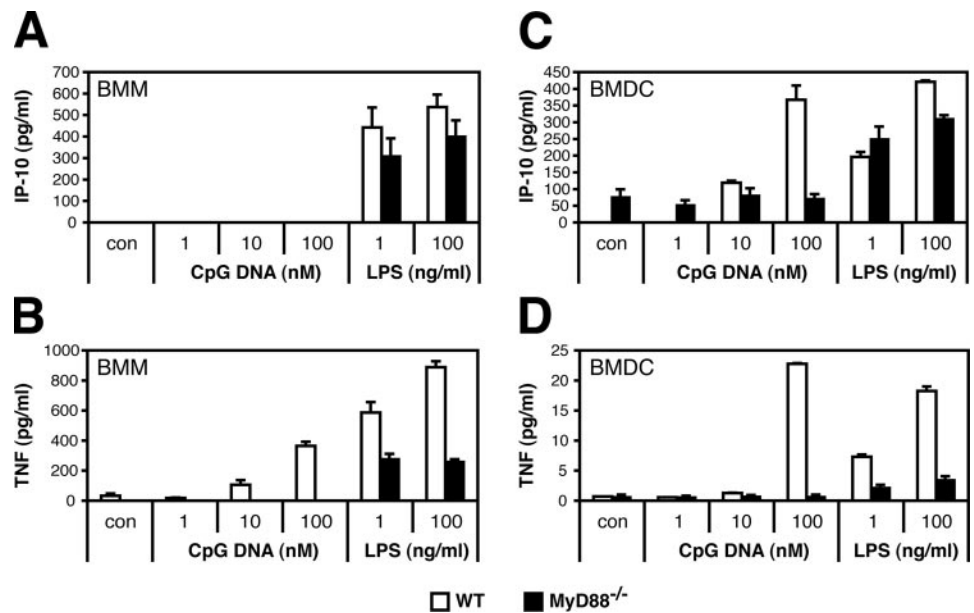
(35), and whole cell and fractionated extracts were analyzed by Western blot, as described previously (35, 36).

## Results

### *CpG DNA induces type I IFN and downstream target genes via MyD88 in mouse DC, but not macrophages*

CpG DNA did not induce IFN- $\beta$  expression in mouse bone marrow-derived macrophages (BMM) or RAW264.7 cells (26), but recent studies have identified a MyD88-dependent pathway leading to *Ifn- $\beta$*  production in BMDC (8, 23, 24). Fig. 1 shows a direct comparison of the effects of the B-type CpG DNA, 1668S, on mouse macrophages and DC. CpG DNA strongly induced *Ifn- $\beta$*

**FIGURE 2.** CpG DNA-induced IFN- $\beta$ -dependent gene expression in BMDC is dependent on MyD88. BMM (A and B) and BMDC (C and D) derived from WT ( $\square$ ) or MyD88 knock-out mice ( $\blacksquare$ ) were stimulated with varying doses of CpG DNA or LPS overnight. Culture supernatants were assayed for secretion of IP-10 (A and C) and TNF (B and D) by ELISA. Data points represent the average of triplicates  $\pm$  SD. Profiles are representative of two independent experiments.



mRNA expression (Fig. 1A), as well as expression of the type I IFN target gene, *Ip-10* (Fig. 1B) in BMDC. In contrast, the mouse macrophage cell line J774 responded to LPS, but not CpG DNA, with inducible *Ifn- $\beta$*  mRNA expression (Fig. 1C), as well as downstream responses such as induction of *Ip-10* mRNA (Fig. 1D) and protein (data not shown). The responsiveness of J774 cells to both stimuli was confirmed by assessing *Tnf* mRNA expression (data not shown). Similarly, stimulation of BMM with LPS, but not CpG DNA, for 3 h elicited robust *Ifn- $\beta$*  mRNA expression (Fig. 1E). In these cells, the TLR2 ligands, Pam<sub>3</sub>CSK<sub>4</sub> and LTA, were also ineffective at inducing *Ifn- $\beta$*  gene expression (Fig. 1E). Even after prolonged incubation with CpG DNA, there was no induction of *Ifn- $\beta$*  (Fig. 1F), whereas *Il-12p40* (Fig. 1G) and *Tnf* (data not shown) were highly induced.

Distinct classes of CpG-containing oligonucleotides vary in their capacity to elicit type I IFN production. In pDC, A-type oligonucleotides such as D19 and 1585 are a more potent stimulus for *Ifn- $\beta$*  expression than B-type oligonucleotides (26, 37). In BMM, however, neither A-type nor B-type oligonucleotides induced robust *Ifn- $\beta$*  expression (Fig. 1H) or expression of the IFN- $\beta$  target gene *Ip-10* (Fig. 1I). The activity of A-type oligonucleotides was confirmed by assessing *Tnf* expression (Fig. 1J). Because the nature of the CpG DNA did not affect *Ifn- $\beta$*  induction and the B-type oligonucleotide was a more potent activator of macrophage function (Fig. 1J), the B-type CpG DNA 1668S was used for all further studies.

The response to CpG DNA and other TLR agonists is dependent upon the adaptor protein MyD88. Although LPS elicited IP-10 production in both BMM (Fig. 2A) and BMDC (Fig. 2C) from MyD88-deficient mice, production of IP-10 in response to CpG DNA was absolutely dependent on MyD88 in BMDC. As expected, TNF production from both BMM (Fig. 2B) and BMDC (Fig. 2D) was either partially dependent (LPS) or totally dependent (CpG DNA) on MyD88. Hence, CpG DNA-triggered IFN- $\beta$  production in the myeloid DC lineage is MyD88 dependent. By extension, because macrophages can respond to CpG DNA (and TLR2 agonists) in a MyD88-dependent manner, the signaling blockade in these cells lies specifically within the pathways linking MyD88 to IFN- $\beta$  production.

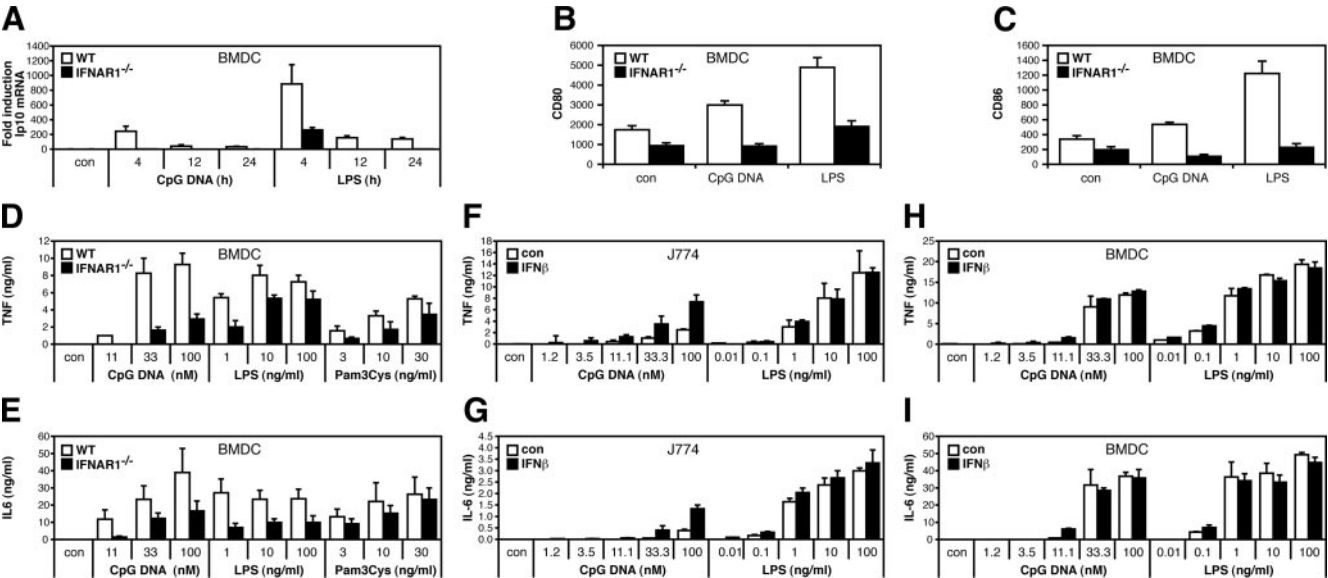
*The type I IFN pathway is required for both cytokine production and cell surface costimulatory molecule expression in response to CpG DNA in BMDC*

Autocrine actions of CpG DNA-induced IFN- $\beta$  have not been widely implicated in DC maturation. We assessed the phenotype of BMDC derived from mice in which the IFN- $\alpha\beta$  receptor is nonfunctional due to targeted deletion of the receptor subunit IFNAR1. As expected, induction of *Ip-10* mRNA by LPS and CpG DNA was impaired in BMDC from IFNAR1-deficient animals (Fig. 3A). CpG DNA- and LPS-mediated up-regulation of surface expression of the costimulatory molecules CD80 (Fig. 3B) and CD86 (Fig. 3C), as well as production of both TNF (Fig. 3D) and IL-6 (Fig. 3E), was also dependent on type I IFN signaling. However, this pathway did not play a major role in the response to Pam3Cys (Fig. 3, D and E).

The *Tnf* and *Il-6* genes are classically regarded as primary response genes in TLR signaling. Because CpG DNA induced *Ifn- $\beta$*  mRNA expression in BMDC, but not macrophages (Fig. 1), we predicted that exogenous IFN- $\beta$  would enhance TNF and IL-6 production in response to CpG DNA in macrophages, but not DC. This was indeed the case (Fig. 3, F–I). As expected, IFN- $\beta$  did not amplify BMM (Fig. 3, F and G) or BMDC (Fig. 3, H and I) responses to LPS, because this agonist induced *Ifn- $\beta$*  mRNA expression in both cell types (Fig. 1). Thus, autocrine IFN- $\beta$  allows DC, but not macrophages, to elicit maximal cytokine production and costimulatory molecule expression in response to CpG DNA.

*CpG DNA activates serine, but not tyrosine, phosphorylation of STAT1 in BMM*

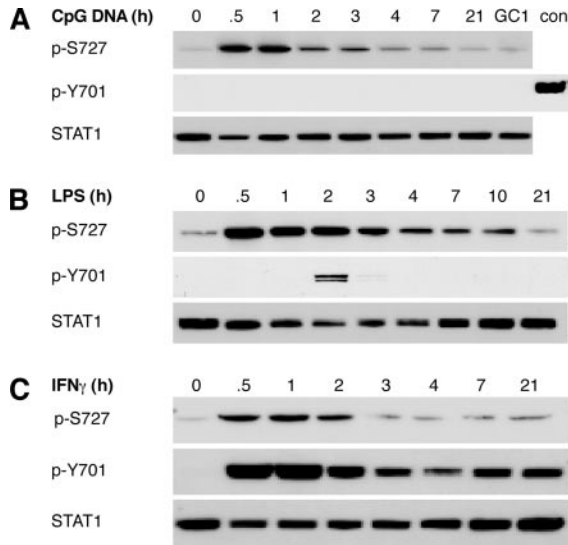
TLR agonists activate STAT1 phosphorylation on S727 via a p38-dependent pathway (13). Since IFN- $\beta$  was not produced in response to CpG DNA in macrophages (Fig. 1), CpG DNA induced an alternative STAT1 activation state in which S727, but not Y701, was phosphorylated in BMM (Fig. 4A). In contrast, both LPS (Fig. 4B) and IFN- $\gamma$  (Fig. 4C) triggered dual Y701/S727 phosphorylation of STAT1 in BMM, as previously reported (38–40). The time course of Y701 phosphorylation in response to LPS was delayed, reflecting the autocrine nature of IFN- $\beta$  signaling.



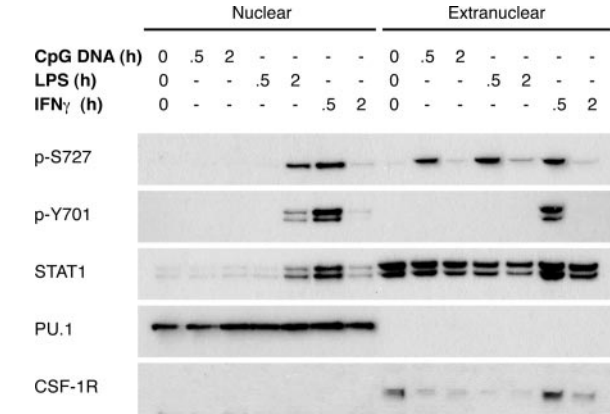
**FIGURE 3.** CpG DNA-induced cytokine production and induction of costimulatory molecules are dependent on type I IFN in BMDC. BMDC derived from WT (□) or IFNAR1 knockout mice (■) were stimulated with 1  $\mu$ M CpG DNA or 100 ng/ml LPS in matched samples over a 24-h time course. Total cellular RNA was prepared, and mRNA expression of *Ip-10* (A) relative to  $\beta$ -actin was determined by qPCR. Data points represent the average of duplicates + SD. Profiles are representative of at least two independent experiments. WT or IFNAR1 BMDC were stimulated with 1  $\mu$ M CpG DNA or 100 ng/ml LPS for 20 h. Surface expression of CD80 (B) and CD86 (C) was analyzed by FACS. Data points represent the average of three quantitations using cells derived from separate mice + SD. Profiles are representative of three independent experiments. WT or IFNAR1 knockout BMDC (D and E), J774 macrophages (F and G), or BMDC (H and I) were stimulated with varying doses of CpG DNA or LPS overnight, in the presence or absence of 100 U/ml IFN- $\beta$  (F-I). Culture supernatants were harvested and assayed for TNF (D, F, and H) and IL-6 (E, G, and I) secretion by ELISA. Data points represent the average of duplicate quantitations + SD. Profiles are representative of three independent experiments.

STAT1 homo- and heterodimerization are mediated by phosphorylation of Y701 at the C-terminal end of the STAT1 Src homology 2 domain (41). Fig. 5 shows that STAT1 translocated to the nucleus during classical activation (Y701, S727 diphosphorylation) by LPS or IFN- $\gamma$ , but was retained in the cytoplasm fol-

lowing alternative activation (monophosphorylation at S727) by CpG DNA. The integrity of the nuclear and extranuclear extracts used in this analysis was confirmed by immunoblotting for PU.1 (a nuclear transcription factor) and the exclusively extranuclear protein, CSF-1R (a plasma membrane receptor). Total STAT1 levels served as an internal control within the experiment. We and others showed that LPS and CpG DNA caused a loss of cell surface CSF-1R due to cleavage of the extracellular domain, whereas IFN- $\gamma$  only modestly down-regulated cell surface CSF-1R expression (34, 36, 42, 43). Consistent with these previous findings,

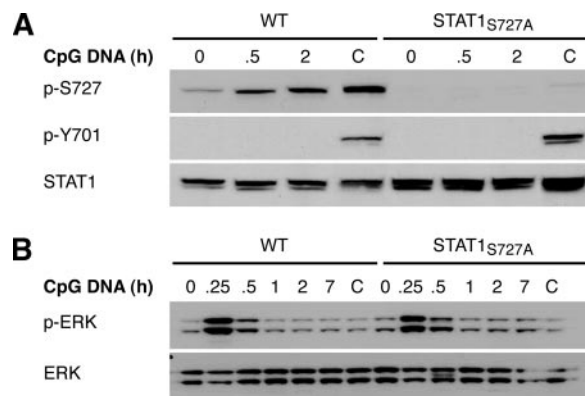


**FIGURE 4.** CpG DNA triggers S727, but not Y701, STAT1 phosphorylation in BMM. BMM were treated with A, CpG DNA or the GC inversion of the CpG DNA oligonucleotide (GC, for 1 h) to control for CpG DNA-independent effects; B, LPS; or C, IFN- $\gamma$  over a time course. Phospho-S727 STAT1 (p-S727), phospho-Y701 STAT1 (p-Y701), and total STAT1 in whole cell extracts were detected by immunoblotting. A positive control (con, 0.5-h IFN- $\gamma$  stimulation) was included for the detection of phospho-Y701 STAT1 on blots containing CpG DNA time courses. Profiles are indicative of at least three independent experiments.



**FIGURE 5.** S727-phosphorylated STAT1 is retained in the cytoplasm following CpG DNA stimulation. BMM were treated with CpG DNA, LPS, or IFN- $\gamma$ , and the cellular localization of STAT1 was monitored. The nuclear and extranuclear compartments were fractionated, and levels of phospho-S727 STAT1 (p-S727), phospho-Y701 STAT1 (p-Y701), total STAT1, PU.1, and CSF-1R in the nuclear and extranuclear cell extracts were detected by immunoblotting. Phospho-STAT1, total STAT1, and PU.1 profiles are representative of at least two independent experiments.





**FIGURE 6.** CpG DNA-induced ERK phosphorylation is intact in STAT1<sub>S727A</sub> BMM. **A**, BMM from WT and STAT1<sub>S727A</sub> mice were stimulated with CpG DNA over a 2-h time course in matched samples. Levels of phospho-S727 STAT1 (p-S727), phospho-Y701 STAT1 (p-Y701), and total STAT1 in whole cell extracts were detected by immunoblotting. A positive control for Y701 STAT1 phosphorylation (C, 0.5-h IFN- $\gamma$  stimulation) was included in the time course. **B**, BMM from WT and STAT1<sub>S727A</sub> mice were stimulated with CpG DNA over a 7-h time course in matched samples. Cells were also treated with the GpC DNA-negative control oligonucleotide (C, 0.25-h GpC DNA). Levels of phospho-ERK-1/2 (p-ERK) and total ERK-1/2 were determined by immunoblotting.

CSF-1R levels in extranuclear extracts were markedly reduced in response to LPS and CpG DNA, but were only modestly affected by IFN- $\gamma$  (Fig. 5), thus confirming the activity of all stimuli used. Hence, BMM responding to CpG DNA provide a model system in

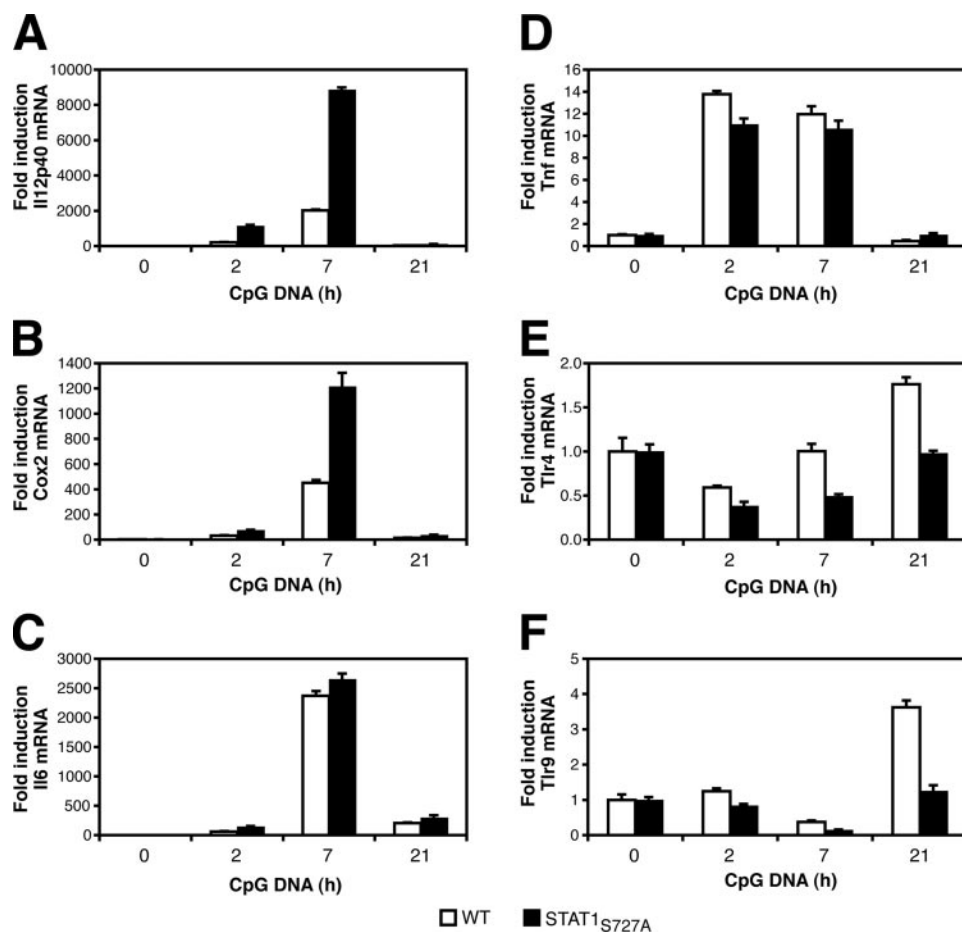
which the function of S727 phosphorylation can be examined in isolation.

#### Alternatively activated STAT1 regulates gene expression in response to CpG DNA in BMM

The function of STAT1 S727 phosphorylation in the CpG DNA response was examined using mice in which the S727 residue was replaced with an alanine (STAT1<sub>S727A</sub> mice) (12). BMM from these mice showed a selective defect in serine phosphorylation in response to CpG DNA or IFN- $\gamma$ , whereas IFN- $\gamma$ -dependent Y701 phosphorylation was intact (Fig. 6A). STAT1-independent signaling by CpG DNA was unaffected by the S727 mutation, because ERK-1/2 activation in response to CpG DNA was comparable between WT and STAT1<sub>S727A</sub> BMM (Fig. 6B).

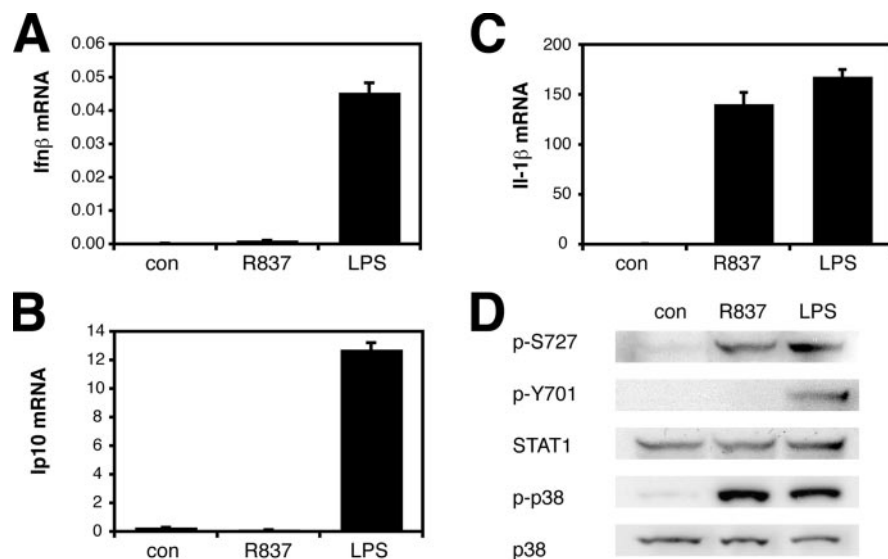
Given that CpG DNA activated the type I IFN pathway in DC, but not macrophages (Fig. 1), we predicted that STAT1, phosphorylated on S727, but not Y701, would have a unique function in macrophages. This was examined by comparing the expression of known CpG DNA-responsive genes in BMM from WT and STAT1<sub>S727A</sub> mutant animals. Induction of both *Il-12p40* and *Cox-2* mRNAs was enhanced 3- to 4-fold in BMM from the mutant mice (Fig. 7, A and B), whereas the induction of *Il-6* and *Tnf* mRNA by CpG DNA was unaffected (Fig. 7, C and D). CpG DNA also selectively represses expression of some genes, including certain receptors and signal transducers. Both *Tlr4* and *Tlr9* mRNAs were transiently repressed by CpG DNA in WT BMM, and were hyperrepressed in STAT1<sub>S727A</sub> BMM (Fig. 7, E and F). Hence, phosphorylation of STAT1 at S727 constrains both the induction (*Il-12p40*, *Cox-2*) and the repression (*Tlr4*, *Tlr9*) of certain genes in response to CpG DNA. The data therefore support a regulatory

**FIGURE 7.** CpG DNA-modulated gene expression is selectively dysregulated in STAT1<sub>S727A</sub> BMM. BMM from WT (□) and STAT1<sub>S727A</sub> mice (■) were stimulated with CpG DNA over a 21-h time course. Total cellular RNA from matched samples was extracted, and mRNAs for *Il-12p40* (A), *Cox-2* (B), *Il-6* (C), *Tnf* (D), *Tlr4* (E), and *Tlr9* (F) were measured by quantitative PCR. Expression is shown as fold induction over basal expression within the genotype. Profiles are representative of three independent experiments.





**FIGURE 8.** R837 triggers S727, but not Y701, STAT1 phosphorylation in HMDM. HMDM were treated with 10  $\mu$ g/ml R837 or 10 ng/ml LPS for 2 h. A–C, Total cellular RNA was prepared, and mRNA expression of *Ifn- $\beta$*  (A), *Ip-10* (B), and *Il-1 $\beta$*  (C) relative to *Hprt* was determined by qPCR. Data points represent the average of triplicates  $\pm$  SD. D, Phospho-S727 STAT1 (p-S727), phospho-Y701 STAT1 (p-Y701), total STAT1, phospho-p38 MAPK (p-p38), and total p38 MAPK in whole cell extracts were detected by immunoblotting. RNA and protein profiles are indicative of duplicate experiments from independent blood donors.



role for serine-phosphorylated STAT1 in limiting macrophage responses to CpG DNA.

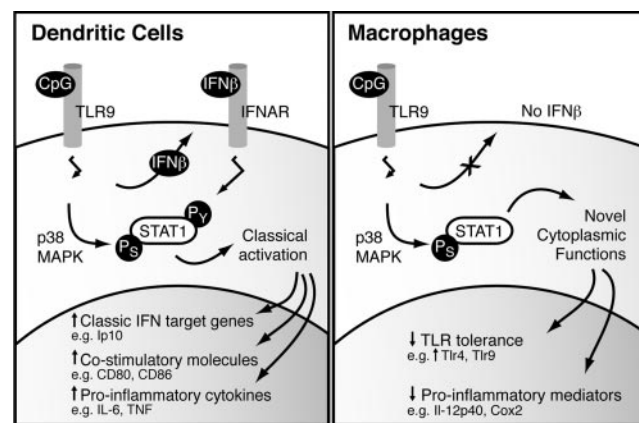
#### A TLR7 agonist triggers alternative STAT1 activation in primary human monocyte-derived macrophages (HMDM)

It is generally accepted that human monocytes do not respond to CpG DNA, which might imply that STAT1 cannot be alternatively activated in human monocytes or macrophages. We hypothesized, however, that any TLR agonist that activates the p38 MAPK pathway without concomitant induction of autocrine IFN- $\beta$  signaling would initiate alternative STAT1 activation in human macrophages. We therefore examined the activation of this pathway in response to the TLR7 agonist, R837 (imiquimod) (27, 28), in HMDM. Although LPS strongly induced *Ifn- $\beta$*  mRNA as expected, R837 was ineffective at inducing *Ifn- $\beta$*  mRNA (Fig. 8A) or the IFN- $\beta$  target gene *Ip-10* (Fig. 8B) in HMDM. In contrast, this signal triggered robust *Il-1 $\beta$*  mRNA expression (330-fold, Fig. 8C) and p38 MAPK phosphorylation (Fig. 8D). Consistent with the ability of R837 to activate p38 MAPK, but not IFN- $\beta$  signaling (Fig. 8, A, B, and D), R837 elicited alternative STAT1 activation (monophosphorylation at S727), whereas LPS triggered classical STAT1 activation (S727 and Y701 phosphorylation) in HMDM (Fig. 8D). Thus, alternative and classical STAT1 activation occur in both mouse and human primary macrophages.

#### Discussion

Although *Ifn- $\beta$*  was initially identified as a MyD88-independent target gene in TLR3 and TLR4 signaling pathways in macrophages, CpG DNA induces this gene in a MyD88-dependent fashion in pDC (25, 26) and myeloid DC (23, 24). We confirmed that myeloid DC and macrophages are different in this respect; CpG DNA induced *Ifn- $\beta$*  mRNA expression as well as IFN- $\beta$  target genes in a MyD88-dependent fashion only in BMDC (Figs. 1 and 2) (23, 24). Although A-type oligonucleotides such as D19 and 1585 are reportedly a much more powerful stimulus for type I IFN production than B-type oligonucleotides in pDC (26, 37), we found that BMDC responded robustly to the B-type oligonucleotide 1668S in this respect (Fig. 1), consistent with the recent findings of Schmitz et al. (23). This pathway was important for myeloid DC maturation and function, because maximal TNF and IL-6 production and costimulatory molecule expression in response to CpG DNA were dependent upon autocrine IFN signaling (Fig. 3).

In macrophages, even A-type oligonucleotides were ineffective at inducing *Ifn- $\beta$*  mRNA expression (Fig. 1H) and downstream IFN target genes (Fig. 1I). The endosomal trafficking of A-type oligonucleotides leading to IRF7-dependent induction of type I IFN (26) is likely to be unique to the antiviral function of pDC. The use of the autocrine type I IFN pathway in DC, but not macrophages, may contribute to the enhanced capacity of DC to act as professional APC, and implies that endogenous IFN contributes to functional divergence between macrophages and myeloid DC. The differential responses of DC and macrophages to CpG DNA are summarized in Fig. 9.



**FIGURE 9.** Model for divergent STAT1 activation and function in response to CpG DNA in DC vs macrophages. CpG DNA stimulation of DC causes STAT1 phosphorylation at both the S727 and Y701 residues. S727 phosphorylation occurs rapidly (within 30 min) and is mediated by a p38 MAPK-dependent pathway. Y701 phosphorylation occurs in a delayed fashion ( $\sim$ 2 h), and is mediated by the autocrine activity of CpG DNA-induced IFN- $\beta$ . Y701/S727-double phosphorylated STAT1 dimerizes and translocates to the nucleus to *trans* activate classic STAT1 target genes such as *Ip-10* and to induce costimulatory molecule expression and proinflammatory cytokine production. CpG DNA stimulation of macrophages triggers phosphorylation of STAT1 at the S727 residue only. In contrast to DC, CpG DNA does not induce the production and autocrine activities of IFN- $\beta$  in macrophages, and so STAT1 remains in the Y701-unphosphorylated form that cannot dimerize or translocate to the nucleus. Cytoplasmic STAT1 indirectly modulates gene expression (e.g., *Tlr4*, *Tlr9*, *Cox-2*, *Il-12p40*), perhaps by cytoplasmic sequestration of gene regulatory factors.

The mechanisms responsible for the divergent effects of CpG DNA on IFN- $\beta$  expression in DC vs macrophages may be linked to differential expression or function of the IRF transcription factor family. The induction of IFN- $\beta$  by CpG DNA requires IRF7 in pDC (25, 26) and IRF1 in BMDC (23, 24). In BMM, IFN- $\gamma$  induced IRF1 mRNA expression and enabled the assembly of the MyD88-dependent pathway leading to IFN- $\beta$  production in response to CpG DNA (23). Thus, a likely explanation is that either IRF1 expression or function is limiting in resting macrophages. However, differential expression of other IRF family members might also explain the absence of the MyD88-IFN- $\beta$  pathway in this cell type. We recently showed that the mRNA expression of IRF4, as well as IRF8 that has been implicated in CpG DNA-specific signaling in DC (44), was down-regulated by the macrophage growth factor, CSF-1, in BMM (34). A prediction of this finding is that these factors would be more highly expressed in DC than in macrophages. Certainly, IRF4 and IRF8 are functionally required for DC differentiation in response to GM-CSF and FLT-3, respectively (45). We are currently characterizing the expression of the entire IRF family in macrophages vs BMDC to identify differentially expressed family members.

Although CpG DNA did not activate the classical type I IFN-dependent pathway in BMM, the phosphorylation of STAT1 on S727 (Fig. 4) led to an alternative activation state in which STAT1 was retained in the cytoplasm (Fig. 5). Gene expression profiles comparing WT and S727A STAT1 mutant macrophages (Fig. 7) suggest that phosphorylation on this amino acid contributes to CpG DNA-mediated signaling in macrophages. Serine phosphorylation of STAT1 regulates interactions with several binding partners (e.g., BRCA1, CBP, MCM5 (12, 46–48)). On this basis, we propose that S727-phosphorylated STAT1 functions to sequester transcriptional regulators in the cytoplasm of CpG DNA-stimulated macrophages. Such a mechanism was demonstrated in osteoblasts, where serine-phosphorylated STAT1 sequestered the RUNX2 transcription factor in the cytoplasm in an inducible manner that was independent of Y701 phosphorylation (49).

One possibility is that members of the IRF family could be targeted for cytoplasmic sequestration by S727-phosphorylated STAT1 in macrophages. For example, IRF9, which normally resides in both the nucleus and cytoplasm, was retained in the cytoplasm when coexpressed with STAT2 (50). The IRF9 nuclear localization signal is contained within the DNA binding domain and is conserved in IRF8 and IRF4 (50), and it is possible that these IRFs could be inducibly retained by STAT1 in the cytoplasm of macrophages, upon S727 phosphorylation. In support of such a mechanism, IRF8 was reported to bind to nontyrosine-phosphorylated STAT1 (51). Other IRF family members are also candidates. IRF1, which was reported to interact with STAT1 (17), positively regulated the inducible expression of *Cox-2* (52) and *Il-12-p40* (53), both of which were superinduced by CpG DNA in STAT1<sub>S727A</sub> BMM (Fig. 7). Similarly, IRF2, which can also form a complex with STAT1 (54), was implicated in driving *Il-12p40* expression (53). STAT1<sub>S727A</sub> BMM may therefore permit the nuclear translocation of one or more IRF family members and the superinduction of *Cox-2*, *Il-12-p40*, and coregulated genes in response to CpG DNA; we are currently investigating this hypothesis.

The differing activity profiles of STAT1 in response to CpG DNA and LPS may underpin their very different toxicities. IFN- $\beta$  signaling and classic STAT1 *trans*-activator function in macrophages contributes to LPS-mediated toxicity in vivo (55, 56). CpG DNA is less toxic than LPS in vivo, and the apparently anti-inflammatory actions of alternatively activated STAT1 may be protective in this context.

Alternative STAT1 activation was not just restricted to CpG DNA signaling in mouse macrophages. The TLR7 agonist, R837, induced a similar activation profile (monophosphorylation at S727) due to a failure of autocrine IFN- $\beta$  signaling in HMDM (Fig. 8). It is likely that a range of stimuli that activate the p38 MAPK pathway without inducing autocrine IFN signaling trigger alternative STAT1 activation. Furthermore, this pathway may also operate as a feedback regulator of TLR4 signaling because LPS triggers sustained S727 phosphorylation, but transient Y701 phosphorylation (Fig. 4). That is, a shift from classical to alternative STAT1 function after prolonged activation via TLR4 may act to sequester transcriptional activators in the cytoplasm.

In summary, we propose that the IFN- $\beta$ /STAT1 axis is a key regulator of CpG DNA signaling, and that divergent activation of this pathway between macrophages and DC dictates cell-specific responses to CpG DNA. CpG DNA induces autocrine IFN- $\beta$  signaling and classic STAT1 transcription factor activities in myeloid DC, thereby potentiating expression of IFN-target genes, costimulatory molecules (CD80 and CD86), and proinflammatory cytokines (IL-6, TNF). The lack of this autocrine pathway in CpG DNA-stimulated macrophages enables alternative STAT1 activities that may include sequestration of gene regulatory factors to limit CpG DNA responses. This blockade could be overcome by IFN- $\gamma$ , which can induce STAT1 Y701 phosphorylation and nuclear translocation. In such a scenario, STAT1 would be rapidly transformed from signaling repressor to transcriptional activator to elicit appropriate antimicrobial responses.

## Disclosures

The authors have no financial conflict of interest.

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